



**UNIVERSITI PUTRA MALAYSIA**

**ENRICHMENT OF OLEIC ACID IN PALM OLEIN HYDROLYSATE  
THROUGH SELECTIVE HYDROLYSIS USING MYCELIUM-BOUND  
LIPASE FROM AN INDIGENOUS *GEOTRICHUM CANDIDUM***

**LOO JOO LING**

**FSTM 2007 7**



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**By**

**LOO JOO LING**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,  
in Fulfilment of the Requirement for the Degree of Doctor of Philosophy**

**April 2007**



## **DEDICATION**

Specially Dedicated to...

### **My Beloved**

#### **Husband and Child**

Kenny Hooi and Jia Yi

### **Late Father**

Mr. Loo Swee Seng

### **Mother**

Madam Kua Poh Yok

### **Brothers and Sisters**

Jo Lee, Joo Teng, Lip Keong, Joo Yee and Lip Wei

Together, We Soar for Success



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

**ENRICHMENT OF OLEIC ACID IN PALM OLEIN HYDROLYSATE THROUGH SELECTIVE HYDROLYSIS USING MYCELIUM-BOUND LIPASE FROM AN INDIGENOUS *GEOTRICHUM CANDIDUM***

By

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**April 2007**

**Chairman: Professor Hasanah Mohd. Ghazali, PhD**

**Faculty: Food Science and Technology**

The lipase of *Geotrichum candidum* is highly specific for unsaturated acyl esters with a *cis*-9 double bond. The preferential reactions for *cis*-9 unsaturated fatty acids (UFA) can be utilised for the enrichment of *cis*-9 UFA notably oleic and linoleic acids in palm olein hydrolysates. Thus, this study was conducted with the aims to isolate a lipolytic microfungus from local soil, and subsequently to study the production, properties, catalytic performance and applications of its mycelium-bound lipase (MBL) in selective hydrolysis of oleic and linoleic acids. MBL besides having an edge over chemical catalyst, offers great potential in industrial application particularly in cost reduction. A strain of *G. candidum*, isolated from local soil, was determined to produce lipase that hydrolyses palm olein in the selective oil agar and liquid medium. The growth of *G. candidum* was studied over a 5-day incubation period. Maximum dry mycelium weight (12.2 g/L) and highest lipolytic activity (7019.5 U/g) was obtained after 96 and 54 hours, respectively, in medium inoculated with 2% (v/v) of 24-hour seed culture and contained 0.1% (w/v) peptone, 0.2% glucose, 2% yeast-extract, 0.1% dipotassium hydrogen phosphate, 0.5% ammonium



sulphate and 2% sterilised palm olein when the culture was incubated at 30°C, at initial culture pH of 7.2. The increase in mycelium mass was concomitant with depletion in triacylglycerols (TAG) and accumulation of free fatty acid (FFA) in the medium, with maximum FFA (90.5%) being detected after 48 hours. Using spore suspension as an inoculation method was not favourable since lower yield of mycelia mass was obtained and the culture exhibited relatively low lipolytic activity and efficiency of hydrolysis.

Large scale cultivation of *G. candidum* revealed that the production and activity of MBL to be dependent on incubation time, where prolonged incubation resulted in secretion of bound lipase into the culture medium. Therefore, all MBL were harvested during early phase of growth at 54 hours when optimal lipase activity was detected. MBL from *G. candidum* demonstrated a high preference for esters with a double bond at *cis-9* position even in crude form. The activity and specific lipase activity of the MBL were, on the average, 22.59 U/g lipase powder and 510 U/g protein, respectively. However, these activities were lower compared to commercial preparations of lipases like Lipozyme IM60 lipase from *R. miehei* and Lipase A from *A. niger*. Reproducibility of MBL from *G. candidum* was possible since the replicate batches of MBL exemplified similar catalytic properties, regio-, and substrate selectivities as its purified, externally immobilised counterpart. Study on the effect of storage on the stability of MBL showed that its activity degraded by 30% after eight months of storage.

MBL of *G. candidum* was found to achieve optimum selective hydrolysis of refined, bleached and deodorised (RBD) palm olein at 3% (w/w) lipase concentration with



90% (w/v) olein-in-*n*-hexane and 60% (v/v) phosphate buffer at pH 7.2 in 30°C water bath with shaking at 200 rpm for 24 hours. It was shown that the degree of discrimination of MBL towards unsaturated substrate was not affected by reaction conditions, but the degree of hydrolysis of these substrates was affected by reaction conditions. In Lipozyme-catalysed hydrolysis of palm olein, the highest hydrolysis degree was found in reaction composed of 90% olein-in-*n*-hexane, 2% lipase and 60% buffer at 60°C. Poor catalytic activity was detected at temperature lower than 50°C and at lower (below 60%) water content. Likewise, the optimum hydrolysis conditions for the *A. niger* lipase (Lipase A) was observed when reaction was carried out in 75% olein-in-*n*-hexane and 20% buffer with 2% lipase at 30°C. The unique specificity of *G. candidum* lipase for oleic and linoleic acids were demonstrated by their higher content in the FFA fraction following hydrolysis compared to starting oil material. Lipozyme IM60 lipase exerted almost equal reactivity towards palmitic or oleic and linoleic acids while Lipase A showed better reactivity to palmitic acid than oleic and linoleic acids.

Several types of vegetable and seed oils were reacted with MBL, Lipozyme IM60 and Lipase A to investigate the levels of enrichment of oleic and linoleic acids in the FFA fraction. For MBL, the highest extent of hydrolysis was found in palm olein while canola oil was least hydrolysed. In contrast to *G. candidum*, the lipase from *R. miehei* and *A. niger* hydrolysed best canola oil while *Moringa oleifera* seed oil was least hydrolysed. The amounts of FFA produced was not in tandem to the degree of hydrolysis as substantial amounts of monoacylglycerols (MAG) and diacylglycerols (DAG) were also present as intermediary products during the course of hydrolysis. The concentrations of oleic and linoleic acids in the FFA fraction from borage oil

and palm olein were greater by 51.4% and 56.3% following hydrolysis by MBL from *G. candidum*. The effect of lipase selectivity on enrichment of oleic and linoleic acids are more significant in oils with lower monoenes contents but ineffective in oils that readily contained high concentrations of oleic acid (59~76%) such as *Moringa oleifera* seed oil, olive oil and canola oil.

When palm olein was hydrolysed using *G. candidum* lipase on a larger scale, oleic acid was selectively enriched from 42.7% (in starting substrate) to 75.0% accompanied by a decrease in palmitic acid from 39.4% to 16.6% in the FFA fraction. Fractional crystallisation of this FFA fraction with acetone separated it into liquid and solid fractions. By combining selective hydrolysis and fractional crystallisation at sub-zero degrees, the oleic acid content in the liquid fraction was increased further to 80.3 wt% with 160.1% recovery compared to the initial content of the fatty acid in palm olein. The purity of this oleic acid-enriched fraction was very close to the commercial 70%-75% oleic acid produced via chemical hydrolysis method. Differential scanning calorimeter (DSC) analysis showed that the thermal behaviour of the liquid and solid fractions was more distinct when lower crystallisation temperature and higher acetone ratio were used. The presence of higher oleic and linoleic acids in the FFA fractions significantly lowered the melting points of the liquid fraction which also indicated a higher purity of oleic acid being obtained.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia  
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**PERKAYAAN ASID OLEIK DALAM HIDROLISAT OLEIN KELAPA  
SAWIT MELALUI HIDROLISIS TERPILIH DENGAN MENGGUNAKAN  
LIPASE TERSEKAT-MISELIA DARIPADA *GEOTRICHUM CANDIDUM*  
TEMPATAN**

Oleh

**LOO JOO LING**

**April 2007**

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Lipase daripada *Geotrichum candidum* mempunyai kekhususan yang tinggi terhadap ester asid tak tepu yang mengandungi ikatan dubel *cis*-9. Kecenderungan tindakbalas terhadap asid lemak tak tepu *cis*-9 ini boleh digunakan untuk memperkayakan asid lemak tak tepu *cis*-9 terutamanya asid oleik dan linoleik dalam hidrolisat olein kelapa sawit. Oleh yang demikian, kajian ini bertujuan untuk memencilkan sejenis kulat mikro lipolitik dari tanah tempatan, dan seterusnya mengkaji penghasilan, ciri-ciri, daya pemangkinan dan penggunaan lipase tersekat-miselia (MBL) daripadanya dalam hidrolisis asid oleik dan linoleik secara terpilih. Selain daripada mempunyai lebih keistimewaan berbanding dengan mangkin kimia, lipase tersekat-miselia menawarkan potensi yang tinggi dalam pengurangan kos operasi di dalam industri. *Geotrichum candidum* yang dipencilkan dari tanah tempatan didapati menghasilkan lipase yang menghidrolisis olein kelapa sawit di dalam agar minyak terpilih dan media cecair. Pertumbuhan *G. candidum* telah dikaji selama lima hari tempoh penderaman. Berat maksimum miselia kering (12.2 g/L) and aktiviti lipolitik





maksimum (7019.5 U/g) telah dicapai selepas 96 dan 54 jam, masing-masingnya, dalam media yang mempunyai 2% (i/i) kultur benih 24 jam, 0.1% (b/i) peptone, 0.2% glukosa, 2% ekstrak yis, 0.1% dwipotassium hidrogen fosfat, 0.5% ammonium fosfat dan 2% olein kelapa sawit yang telah disterilkan, apabila kultur dieram pada suhu 30°C dan pH 7.2. Peningkatan berat miselia dalam media adalah sejajar dengan pengurangan triasilgliserol dan peningkatan asid lemak bebas dimana tahap maksimum asid lemak bebas dicapai selepas 48 jam. Penggunaan mendakan spora sebagai kaedah inokulasi adalah kurang sesuai kerana penghasilan jumlah miselia adalah lebih rendah, aktiviti lipolitik and keberkesanan hidrolisis juga turun secara relatif.

Pertumbuhan *G. candidum* secara skala besar menunjukkan penghasilan dan aktiviti lipase tersekat-miselial bergantung kepada masa penderaman dimana penderaman berlanjutan akan menyebabkan lipase tersekat-miselial dibebaskan daripada miselia ke dalam media kultur. Oleh yang demikian, semua lipase tersekat-miselial telah dikutip pada 54 jam, iaitu di peringkat awal fasa tumbesaran dimana aktiviti lipase adalah optimum. Lipase tersekat-miselial daripada *G. candidum* walaupun di dalam bentuk mentah, menunjukkan kecenderungan terhadap ikatan ester yang mempunyai ikatan dubel di posisi *cis*-9. Purata aktiviti dan aktiviti khusus lipase tersekat-miselial adalah 22.5 U/g serbuk lipase dan 510 U/g protein, masing-masingnya. Akan tetapi, aktiviti ini adalah lebih rendah berbanding dengan lipase komersial seperti lipase Lipozyme IM60 daripada *R. miehei* dan Lipase A daripada *A. niger*. Penghasilan berulang lipase terikat-miselial daripada *G. candidum* boleh dilakukan sebab replikasi batch menunjukkan ciri-ciri pemangkinan, pilihan regio dan pilihan substrat yang sama dengan lipase tulen yang tersekat-gerak. Kajian mengenai kesan



penyimpanan ke atas kestabilan lipase terikat-miselia menunjukkan aktiviti akan merosot sebanyak 30% berikutan penyimpanan selama lapan bulan.

Keadaan optima untuk lipase terikat-miselia daripada *G. candidum* menjalankan hidrolisis terpilih ke atas olein kelapa sawit adalah 3% (b/b) kepekatan lipase, 90% (b/i) olein-dalam-*n*-hexane dan 60% (i/i) penimbal fosfat (pH 7.2) pada suhu 30°C dengan putaran 200 rpm selama 24 jam. Didapati darjah diskriminasi lipase terikat-miselia terhadap substrat tak tepu tidak dipengaruhi oleh keadaan tindakbalas, tetapi darjah hidrolisis substrat ini bergantung kepada keadaan tindakbalas. Dalam hidrolisis olein kelapa sawit yang dimangkinkan oleh lipase Lipozyme IM60, darjah hidrolisis tertinggi dicapai pada 90% olein-dalam-*n*-hexane, 2% lipase dan 60% penimbal pada suhu 60°C. Aktiviti pemangkinan yang lemah diperolehi pada suhu yang rendah daripada 50°C dan kandungan air di bawah 60%. Keadaan hidrolisis yang optima untuk lipase daripada *A. Niger* (Lipase A) diperlihatkan apabila tindakbalas berlaku di dalam 75% olein-dalam-*n*-hexane, 20% penimbal dan 2% lipase pada suhu 30°C. Kekhususan unik lipase daripada *G. candidum* terhadap asid-asid oleik dan linoleik telah dibuktikan oleh kandungan yang lebih tinggi kedua-dua asid ini dalam bahagian asid lemak bebas berbanding dengan minyak asal. Lipase Lipozyme IM60 menunjukkan reaktiviti yang hampir sama terhadap asid oleik dan asid linoleik manakala Lipase A menunjukkan reaktiviti yang lebih baik ke atas asid palmitik daripada asid oleik dan linoleik.

Pelbagai jenis minyak sayuran dan bijian telah ditindakbalaskan dengan lipase terikat-miselia, lipase Lipozyme IM60 dan Lipase A bagi mengkaji tahapperkayaan asid oleik dan linoleik dalam bahagian asid lemak bebas. Bagi lipase terikat-

miselia, darjah hidrolisis tertinggi diperolehi dengan olein kelapa sawit manakala minyak kanola paling kurang dihidrolisis. Di sebaliknya, minyak kanola dihidrolisis paling baik oleh lipase daripada *R. miehei* and *A. niger* manakala minyak biji *Moringa oleifera* paling kurang dihidrolisis. Namun asid lemak bebas yang dihasilkan tidak sejajar dengan darjah hidrolisis ini adalah kerana sebahagian besar monoasilgliserol dan diasilgliserol turut dihasilkan sebagai hasil perantaraan semasa tindakbalas hidrolisis. Kepekatan asid oleik dan linoleik dalam bahagian asid lemak bebas daripada minyak borage dan olein kelapa sawit telah berjaya diperkayakan sebanyak 51.4% dan 56.3% berikutan hidrolisis oleh lipase tersekat-miselial daripada *G. candidum*. Kesan pilihan lipase ke atas asid oleik dan linoleik lebih jelas diperlihatkan dalam minyak yang mengandungi kandungan monoene yang rendah tetapi tidak berkesan ke atas minyak yang mengandungi kepekatan asid oleik yang tinggi (59~76%) seperti minyak biji *Moringa oleifera*, minyak zaitun dan minyak kanola.

Apabila olein kelapa sawit dihidrolisis oleh lipase *G. candidum* secara skala besar, asid oleik diperkayakan daripada 42.7% (dalam substrat asal) ke 75.0%, diiringi dengan penurunan kandungan asid palmitik daripada 39.4% ke 16.6% dalam bahagian asid lemak bebas. Pembekuan pemeringkatan bercampur aseton ke atas bahagian asid lemak bebas ini memisahkannya kepada bahagian cecair dan bahagian pepejal. Dengan menggabungkan kaedah hidrolisis terpilih dan pembekuan pemeringkatan pada suhu di bawah kosong darjah, kandungan asid oleik dalam bahagian cecair dipertingkatkan lagi kepada 80.3% dengan 160.1% pemulihan berbanding dengan kandungan asalnya dalam minyak olein kelapa sawit. Ketulenan bahagian ini yang diperkayakan dengan asid oleik adalah hampir sama dengan 70%-

75% asid oleik komersial yang dihasilkan melalui kaedah hidrolisis kimia. Analisis dengan kalorimeter pengimbangan pembezaan menunjukkan perbezaan yang ketara dalam kelakuan haba di antara bahagian cecair dan pepejal bila suhu pembekuan yang lebih rendah dan nisbah aseton yang lebih tinggi digunakan. Kehadiran asid oleik dan linoleik yang lebih tinggi dalam asid lemak bebas telah menurunkan secara bererti takat lebur bahagian cecair yang diperolehi yang membuktikan ketulenan tinggi bagi asid oleik yang diperolehi.

## ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my chief supervisor, Prof. Dr. Hasanah Mohd. Ghazali, for her invaluable guidance, support and constructive suggestions throughout the course of my graduate study and for her editorial assistance during the preparation of this dissertation. My grateful thanks also go to my co-supervisors, Assoc. Prof. Dr. Lai Oi Ming and Dr. Kamariah Long for their support, advices and encouragement throughout the course of this study.

Many thanks are also due to staffs and research assistants of the Faculty of Food Science and Technology, Faculty of Biotechnology and Biomolecular Sciences and MARDI for their kind cooperation and technical support rendered. Warm appreciations and acknowledgements are also extended to all members of the Enzyme Technology Laboratory, who have made my time spent in the laboratory, a memorable one.

My acknowledgement is also extended to the Government of Malaysia for the IRPA research grant awarded to Prof. Dr. Hasanah Mohd. Ghazali and team, that has enabled me to pursue my PhD. Degree.

Heartfelt appreciation is accorded to my beloved husband for his support, encouragement, patience and sacrifices, which have kept me motivated and made possible the completion of my research and dissertation. Special thanks go to my dearest family, who always has faith and confident in me.



I certify that an Examination Committee has met on 9<sup>th</sup> April 2007 to conduct the final examination of Loo Joo Ling on her Doctor of Philosophy thesis entitled “Enrichment of Oleic Acid in Palm Olein Hydrolysate Through Selective Hydrolysis Using Mycelium-Bound Lipase from an Indigenous *Geotrichum candidum*” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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## **DECLARATION**

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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**LOO JOO LING**

Date: 2 MAY 2007



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